REMARKS

Claims 1, 53, 54, 57, 59, 60, and 120-122 are pending in the application. Claims 1, 53, 59, and 121 have been amended and claims 123-131 have been added. Accordingly, claims 53, 54, 57, 59, 60, and 120-131 will be remain pending in the application upon entry of the claim amendments presented herein.

Claims 1, 53, and 59 were amended to correct antecedent basis. Claim 121 was amended to correct a typographical error. Claim 123-129 have been added to claim more fully the instant invention. Support for the claim amendments can be found throughout the specification and claims as originally filed. In particular, support for claim 123 can be found at least, for example, in Example 5 on page 91 of the specification and in Table 3 on page 104 of the specification. Support for claims 124-129 can be found in original method claims 27-29 and 41-43. No new matter has been added.

Amendment and cancellation of the claims are not to be construed as acquiescence to any of the rejections/objections made in the instant Office Action or in any previous Office Action, and were done solely to expedite prosecution of the application. Applicants hereby reserve the right to pursue the claims as originally filed, or substantially similar claims, in one or more subsequent patent applications.

Election/Restrictions

Applicants note that the restriction requirement in this application was issued on July 21, 2004, before the recent changes in restriction practice were put in place. Specifically, MPEP § 821.04(b) now provides as follows:

> Where claims directed to a product and to a process of making and/or using the product are presented in the same application, applicant may be called upon under 35 U.S.C. 121 to elect claims to either the product or a process. See MPEP § 806.05(f) and § 806.05(h). The claims to the nonelected invention will be withdrawn from further consideration under 37 CFR 1.142. See MPEP § 821 through §821.03. ***However, if applicant elects a claim(s) directed to a product which is subsequently found allowable, withdrawn process claims which depend from or otherwise require all the limitations of an allowable product claim will be considered for rejoinder. All claims directed to a***

> ***nonelected process invention must depend from or otherwise require all the limitations of an allowable product claim for that process invention to be rejoined. Upon rejoinder of claims directed to a previously nonelected process invention, the restriction requirement between the elected product and rejoined process(es) will be withdrawn.*** [Emphasis added.]

The method claims as originally filed had been withdrawn from further consideration directed to non-elected subject matter and were cancelled to expedite prosecution of the application. However, in view of MPEP § 821.04(b), Applicants have added herein method claims 124-131 (corresponding to original method claims 27-29 and 41-43), which depend from and include all the limitations of the elected product (yeast cell claims). Presumably, these new claims will be withdrawn from further consideration as the method claims were not elected. However, upon a finding that the pending elected yeast cell claims are allowable, Applicants respectfully request that method claims 124-131 be rejoined pursuant to the provisions of MPEP § 821.04(b).

Objection to the Oath/Declaration under 37 C.F.R. 1.67(a)

The Examiner objects to the Declaration, Petition, and Power of Attorney for Patent Application filed on June 16, 2000 as allegedly unsigned. Applicants respectfully point out that back in 2000, prior to the advent of Application Data Sheets, it was customary to file concurrently with the application an unexecuted Declaration in order to provide the Patent Office with all the relevant information for the application. However, the Examiner's attention is invited to the compilation of executed declarations filed on March 5, 2001 concurrently with a response to a Notification of Missing Requirements. Although it appears that any one of the declarations is not fully executed, Applicants remind the Examiner that in applications having multiple inventors situated in multiple locations, the MPEP permits submission of a compilation of signed declarations, provided the compilation provides signatures of all the inventors. Such is the situation here and, therefore, this objection should be withdrawn.

Objection to the Drawings

The Examiner objects to the drawings "because tables and sequence listings included in the specification must not be duplicated in the drawings." The Examiner is apparently objecting to Figure 1, which includes several sequences. 37 C.F.R. § 1.83 requires that "tables and **sequence listings** that are included in the specification are, except for applications filed under 35 U.S.C. 371, not permitted to be included in the drawings." Applicants note that although Figure 1 includes a number of sequences, it is not "**a sequence listing**." Figure 1 presents a **sequence alignment** that provides information regarding the sequence identity that exists between the N-terminal regions of Gα subunits and N-terminal sequences of GPA41-Gα hyrid proteins. 37 C.F.R. § 1.83 does not apply to sequence alignments. Moreover, the the sequence alignment is not found in the sequence listing. Thus, the objection to the Drawings should be withdrawn.

Claim Rejections - 35 U.S.C. § 112, First Paragraph

Claims 1, 53, 54, 57 and 120-122 are rejected under 35 U.S.C. § 112, first paragraph as lacking enablement in the specification. Although the Examiner alleges that the full scope of the claims is not enabled by the specification, the Examiner does admit on page 5 of the Office Action that the claims are enabled for the G-protein coupled receptors listed in Example 5 on page 91 of the specification and in Table 3 on page 104 of the specification.

The claims are directed to yeast cells that contain a heterologous G protein-coupled receptor (GPCR) and a chimeric G protein subunit which contains an endogenous yeast Gpa1 subunit in which at least the last four C-terminal amino acids of Gpa1 are replaced with at least the last four C-terminal amino acids of a first heterologous G protein subunit, and in which the N-terminus of said Gpa1 is operably linked to at least the first five N-terminal amino acids of a second heterologous G protein subunit (claims 1 and 120-122); or in which at least the last four C-terminal amino acids of said Gpa1 are replaced with at least the last four C-terminal amino acids of a first heterologous G protein subunit, and in which at least the first five N-terminal amino acids of said Gpa1 are replaced with at least the first five N-terminal amino acids of a second heterologous G protein subunit (claims 53, 54 and 57). Claims 59 and 60 are directed to a chimeric G-protein subunit which

comprises an endogenous Gpa1 subunit in which at least the last four C-terminal amino acids of said Gpa1 are replaced with at least the last four C-terminal amino acids of a first heterologous G protein subunit, and in which the N-terminus of said Gpa1 is operably linked to at least the first five N-terminal amino acids of a second heterologous G protein subunit, where the first and second heterologous G protein subunits are the same or different.

The claims are rejected under 35 U.S.C. § 112, first paragraph, as allegedly lacking enablement based on the following assertions: first, that Applicants have failed to disclose common structural and/or functional characteristics of GPCRs in combination with G-protein subunits; second, that the interaction between GPCRs and G proteins is unpredictable; and third, that Applicants' have failed to enable the use of GPCRs or Gpa1 subunits not specifically described in the specification. Applicants respectfully disagree. For the reasons provided below, the rejection should be withdrawn.

1. GPCRs have characteristic structural and functional features

At the time of filing G protein-coupled receptors were well known in the art and were widely recognized as having a number of identifying structural and functional characteristics. G protein-coupled receptors comprise a chain of amino acids that passes through the cell membrane seven times (page 2, lines 2-5). This characteristic structural feature is the reason that G protein-coupled receptors are commonly referred to as seven-transmembrane receptors (STRs) (page 2, lines 2-5). All G protein-coupled receptors propagate an intercellular signal via G proteins (page 1, lines 24-26). G proteins, which consist of α, β, and γ subunits, are complexed with guonosine diphosphate in the inactive state (page 1, lines 28-30). Ligand binding to the G protein-coupled receptor induces a conformational change that alters its interaction with the G protein and triggers release of GDP and binding of GTP (page 1, lines 26-28). The activated GTP-bound G protein triggers a downstream signal transduction pathway via second messenger signaling that is a function shared by all G protein-coupled receptors (page 1, lines 32-34).

Thus, G protein-coupled receptors are structurally and functionally related proteins characterized by seven membrane-spanning alpha helices, an N-terminal segment on the exoplasmic

face and a C-terminal segment on the cytosolic face of the plasma membrane. Although these receptors are activated by different ligands and may mediate different cellular responses, they all mediate a similar signaling pathway.

All G protein-coupled receptors follow the same functional paradigm. The binding of ligands to the extracellular domain of G protein-coupled receptors induces a conformational change that allows the cytosolic domain of the receptor to bind to a G protein associated with the inner face of the plasma membrane. G proteins consist of three subunits, designated α, β, and γ. This interaction activates the G protein, which then dissociates from the receptor and carries the signal to an intracellular target or "effector." Essentially, dissociation of the G-protein heterotrimer into G_α and $G_{\beta\gamma}$ units transmits the signal that the receptor has bound its ligand.

All G protein-coupled receptor ligand binding activates a G protein, which in turn activates or inhibits an effector that generates a specific second messenger or modulates an ion channel, causing a change in membrane potential. Adenylyl cyclase, which catalyzes the formation of cAMP from ATP, is the best-characterized effector regulated by trimeric G proteins. All adenylyl cyclase isoforms are stimulated by $G_{s\alpha}$, and certain isoforms are inhibited by $G_{i\alpha}$ and $G_{\beta\gamma}$. $G_{s\alpha}$, $G_{i\alpha}$. As a result, adenylyl cyclase is stimulated or inhibited by many different G protein-coupled receptor ligands.

Given their common functionality, G protein-coupled receptors enable different receptor-hormone complexes to modulate the activity of the same effector protein, adenylyl cyclase. In many types of cells, for example, binding of different hormones to their respective receptors induces activation of adenylyl cyclase. In the liver, glucagon and epinephrine bind to different G protein-coupled receptors, but binding of both hormones activates adenylyl cyclase and thus triggers the same metabolic responses. In adipose cells, for example, epinephrine, glucagon, and ACTH all stimulate adenylyl cyclase.

Accordingly, these characteristic structural and functional characteristics allow one skilled in the art to readily identify a polypeptide as a G protein-coupled receptor. The Examiner acknowledged as much at page 8, lines 1-3, where the Examiner stated that "the GPCR protein family share common structures such as transmembrane regions and G-protein interaction regions."

Further evidence that one of skill in the art could readily identify polypeptides as G protein-coupled receptors is provided in the following publications: Khorana, J. Biol. Chem. 267:1-4, 1992 (hereinafter "Khorana"); Stiles, J. Biol. Chem 267:6451-6454, 1992 (hereinafter "Stiles"); and Hosey et al., FASEB J. 6:845-852, 1992 (hereinafter "Hosey") (Exhibits A-C), each of which provides a review of the conserved structural and functional features of G protein coupled receptors.

Khorana describes the "An Emerging Pattern for Structure and Function" of rhodopsin, which is a seven transmembrane receptor (Figure 1)which is coupled to GTP-binding proteins (page 1, left column, first paragraph) and propagates a downstream signal transduction pathway as shown in Figure 2, and as described at page 1, right column, under the heading "Biochemical Aspects of Sensory Functions; and Figure 4. Regarding the structure-function of rhodopsin and other G protein-coupled receptors, Khorana states "It is reasonable to expect that **the structure-function pattern presented above for rhodopsin will apply to all the visual pigments as well as to a majority of the G-coupled receptors**. The conserved sequences in different regions of the receptors support this conclusion (page 4, left column, third full paragraph; emphasis added; citations omitted)."

Further evidence that the structure and function of G protein-coupled receptors were known in the art at the time of filing is provided by Stiles, who provides a review of adenosine receptors. Adenosine receptors are G protein coupled receptors that have a seven-transmembrane domain spanning motif (Figure 1). Like other G protein-coupled receptors, adenosine receptors are coupled to G proteins (Table I) and ligand binding to the adenosine receptor effects downstream signal transduction pathways by regulating, for example, adenylyl cyclase activity (page 6452, right column, under the heading "Receptors Effector Coupling).

Still further evidence that the structure and function of G protein-coupled receptors was known in the art at the time of filing is provided by Hosey. Hosey provides a review of the structure, signaling and regulation of muscarinic cholinergic receptors. Muscarinic receptors are G protein coupled receptors that have seven transmembrane domains (Figure 1). Like other G protein coupled receptors, ligand binding to the various muscarinic receptor subtypes modulates downstream signal transduction pathways, including adenylate cyclase (Figure 2). Regarding

muscarinic receptors, Hosey states "there has been an explosion of information concerning the structure, signaling and regulation of what is now known to be a family of muscarinic receptors. This review discusses the five identified members of the mAChR family and their coupling to the multible G proteins that allow mAChRs to modulate many different types of signal transduction pathways."

As evidenced in each of the afore-mentioned publications, G protein coupled receptors were known in the art at the time of filing to have conserved structural and functional features that would allow one of skill in the art to readily identify polypeptides as G protein coupled receptors. In addition, as evidenced in Applicants' specification, Applicants have identified common structural and functional characteristics of G protein-coupled receptors in combination with G-protein subunits. Accordingly, this basis for the rejection should be withdrawn.

2. G protein subunits and G protein-coupled receptors interact in a predictable fashion

In support of the enablement rejection, the Examiner asserts that interactions between G protein-coupled receptors and G protein subunits are highly unpredictable. Specifically, the Examiner states:

> The signal transduction pathway involving G-protein coupled receptor (GPCR) and G-protein are not completely understood in the art. Although the structure of certain GPCR and G-protein heterotrimeric complex have been solved, the intricate structural interaction between the receptor and the G-protein, as well as the interaction among the various subunits (alpha, beta, and gamma) of the G-protein itself are not clearly defined in the art. (Office action mailed August 24, 2007 page 7, lines 16-21)

The standard for enablement does not require that Applicants understand "the intricate structural interactions" among all of the various components of the G protein-coupled receptor signalling pathway. The proper test of enablement is "whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with the information known in the art without undue experimentation." *Hybritech, Inc. v. Monoclonal Antibodies, Inc.* 802 F.2d. 1318

(Fed. Cir. 1985). Applicants have clearly met this standard by disclosing methods for generating the claimed yeast cells and G protein chimeras.

Proper signal transduction requires that each G protein-coupled receptor interact with only specific subclasses of G proteins (page 67, lines 1-10). Applicants discovered that the coupling specificity of heterologous G protein-coupled receptors expressed in yeast can be enhanced by generating "sandwich chimera" in which Gpa1 sequences are flanked at the amino and carboxy-terminal ends by heterologous Gα sequences (page 69, line 22, to page 72, line 29). Construction of these sandwich chimeric G proteins generally involves replacing C-terminal amino acids of Gpa1 with C-terminal amino acids of a heterologous Gα subunit and either replacing N-terminal amino acids of Gpa1 with N-terminal amino acids of a heterologous Gα subunit or adding N-terminal amino acids of a heterologous Gα subunit to the amino terminal of Gpa1 using standard molecular biological techniques. The coupling specificity of the sandwich chimeric G proteins to a heterologous GPCR is tested in yeast cells using, for example, the Fus1-pHIS3 screening assay described in Example 12 (page 107, line 32, to page 108, line 15). Provided with Applicants specification one skilled in the art could predictably generate the claimed yeast cells and sandwich chimeric G proteins.

To illustrate the alleged unpredictability of the claimed invention, the Examiner cites Lambright, *et al.* (*Nature* 379:311-319, 1996; hereinafter "Lambright"), Brown, *et al.* (WO 99/14344; hereinafter "Brown"), and Busconi, et al. (*Biochem J.* 328:23-31, 1997), each of which purportedly shows the "highly unpredictable nature of combining any GPCRs (derived from various sources) with Gpa1 chimeric (which comprises partial Gα amino acid sequences from various sources) (page 9, lines 5-7)." The Examiner's reliance on these references is misplaced. The cited references merely exemplify the state of the art ***prior*** to Applicants' claimed invention. As acknowledged by the Examiner, the cited references describe various mutant Gα subunits that fail to functionally coupling to heterologous G protein-coupled receptors. These references demonstrate the need for yeast cells having improved coupling with a variety of heterologous GPCRs.

In further support of the unpredictability of the art, the Examiner notes that Applicants' specification discloses several Gpa1 chimeric subunits that do not functionally couple with

particular mammalian receptors. Even if, as the Examiner suggests, not every claimed yeast cell or Gpa1 chimera successfully couples to a heterologous receptor, this does not mean that the present claims are overbroad or non-enabled. The Federal Circuit has long held that it is not necessary for all possible embodiments of a claim to be operative in order for that claim to be enabled. *See Atlas Powder Co. v. E.I. du Pont de Nemours & Co.*, 750 F.2d 1569, 224 USPQ (Fed. Cir. 1984). Thus, this basis for the enablement rejection should also be withdrawn.

3. Routine methods identify compatible G protein-coupled receptors and sandwich chimeric G proteins

Finally, the Examiner asserts that the specification provides insufficient guidance to enable the full scope of the claims and that undue experimentation would be required to determine the compatibility of various G protein-coupled receptors and Gpa1 subunit combinations. In particular, the Examiner states:

> The art has not demonstrated that all the desired GPCR and Gpa1 chimeric can be successfully expressed in yeast cells and would possess the desired signal transduction mechanism. The instant specification and/or claims only provide an invitation to experiment with different combination of GPCR and Gpa1 that may or may not produce the desire recombinant yeast cell. Because the instant specification only provides guidance for only a few examples of yeast recombinant cells that comprises a certain types of GPCR and Gpa1 chimera, undue experimentation would be required to produce the claimed genus of recombinant yeast cells.

Applicants respectfully disagree.

Applicants describe yeast cells comprising a chimeric G protein subunit that functionally integrates a heterologous G protein-coupled receptor into the pheromone response pathway of the yeast cell, such that modulation of the signal transduction activity of the heterologous G protein-coupled receptor by an extracellular signal provides a detectable signal (pages 107 to page 108, under the heading "GPA1-Gq sandwich improves functional activity of a bradykinin-responsive receptor"). In particular, Applicants report that the GPA1-Gq sandwich chimera increased coupling

by the human bradykinin responsive receptor 2 in a functional assay. "Applicants state "ß galactosidase activities were increased 26-fold in yeast cells transformed with Gαq(1-11)-GPA1(6-467)-Gαq(355-359). . ." Applicants also report coupling to heterologous orphan receptors using G protein sandwich chimeras in the Fus1 p-His3 assay and in the lacZ assay (pages 104-106). Results using the Fus1 p-His3 assay are provided at Table 4, page 105, where Applicants show that the mammalian Bombesin receptor subtype 3 and the Bradykinin receptor 2 functionally coupled to the Gαq(1-11)-GPA1(6-467)-Gαq(355-359) sandwich chimera. Results of the lacZ assay are provided at Table 5, pages 105-106, where Applicants show that mammalian heterologous orphan receptors 11, 15, and 16 also functionally coupled to the Gαq(1-11)-GPA1(6-467)-Gαq(355-359) sandwich chimera. Contrary to the Examiner's assertion, Applicants have provided a number of working examples showing that G protein-coupled receptor successfully coupled with GPA1 sandwich chimeras.

Using the methods described in Applicants specification and no more than routine experimentation, a skilled artisan could readily identify those yeast cells expressing sandwich chimeras that functionally couple to a heterologous GPCR. Such screening does not constitute undue experimentation because it could easily be accomplished using ***standard*** techniques for generating and screening recombinant yeast cells as described in Applicants' specification, for example, in Example 12. In analyzing what constitutes undue experimentation, the MPEP (§ 2164.06) cites *In re Wands*, (858 F.2d 731, 8 USPQ2d 1400 (Fed Cir. 1988)):

> The test is not merely quantitative, since ***a considerable amount of experimentation is permissible, if it is merely routine***, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. (emphasis added)

The present situation is, in all important aspects, indistinguishable from the facts in *Wands* in which the Federal Circuit held that the applicant's claim was enabled, despite the necessity for screening, because the process of screening was straightforward. It follows that the present claims are also enabled, even if some screening would be necessary to identify yeast cells expressing Gα subunits that functionally couple to a heterologous GPCR.

Moreover,the Examiner has acknowledged that the level of skill in the art is high. Thus, based on that high level of skill, coupled with the teachings and working examples of Applicants' specification, one skilled in the art can make and use the claimed invention without undue experimentation or burden. There is nothing unusual or difficult about making and screening a number of yeast cells, each carrying a heterologous GPCR and a chimeric G protein to identify those yeast cells in which functional coupling between the GPCR and the G protein has occurred. Given the working examples provided by Applicants and the admonition of the Federal Circuit in *In re Wands* that a considerable amount of routine experimentation is permissible and does not preclude enablement, Applicants submit that the scope of the claims is fully enabled by the specification.

Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejection.

Claim Rejections - 35 U.S.C. § 112, Second Paragraph

Claims 1, 53, 54, 57, 59, 60, and 120-122 are rejected under 35 U.S.C. 112, second paragraph for alleged indefiniteness. Althouhg Applicants disagree with this assertion, Applicants have amended the claims in order to expedite prosecution. In particular, claims 1, 53, 54 and 59 now recite a Gpa1 subunit having C and N terminal amino acids.

The Examiner asserts that the phrase "the signal transduction activity," which occurs at line 15 and 16 of claims 1 and 53, respectively, lacks antecedent basis. Applicants respectfully disagree, and note that antecedent basis is found at line 3 of each of the rejected claims. Accordingly, this basis for the indefiniteness rejection should also be withdrawn.

The rejection of claims 57, 60, and 120-122 is overcome by the amendment of the independent claims from which they depend.

Claim Rejections - 35 U.S.C. § 102

Claims 1, 59, 121, and 122 are rejected as allegedly anticipated by Pausch (WO 95/21925; hereinafter "Pausch"); and claims 1, 53, 59, and 120-122 are rejected as allegedly anticipated by Fowlkes (WO 94/23025; hereinafter "Fowlkes"). Applicants respectfully disagree.

As an initial matter, Applicants note that the rejection of claim 1 as anticipated by Pausch and Fowlkes was originally made in the Office action mailed October 19, 2004 and was withdrawn in the Office action mailed July 8, 2005.

In support of the anticipation rejection, the Examiner asserts that the claimed yeast cells and G protein subunits are purportedly within the scope of G protein subunits described in the prior art. Even if we accept *arguendo* that the claimed yeast cells and G protein subunits are "within the scope" of chimeras described by the prior art, this is not sufficient to serve as an anticipation. It is well-known that a prior genus does not anticipate a later species.

To support a rejection of a claim under § 102, a single prior art reference must describe all of the elements present in the rejected claim, *Scripps Clinic & Research Foundation v. Genentech, Inc.*, 927 F.2d 1565, 18 U.S.P.Q.2d 1001, 18 U.S.P.Q.2d 1896 (Fed. Cir. 1991). A comparison of the claimed invention with each of the cited references demonstrates that neither Fowlkes nor Pausch discloses a yeast cell comprising a Gpa1 subunit having a C-terminus linked to a heterologous C terminus of another G protein subunit and an N-terminus where at least 4 amino acids are replaced by four amino acids from a heterologous G protein subunit as recited in claim 1. In addition, neither of the cited references describes a yeast cell or a Gpa1 subunit where the last four C-terminal amino acids are replaced with at least the last four C-terminal amino acids of a first heterologous G protein subunit, and in which at least the first five N-terminal amino acids of Gpa1 are replaced with at least the first five N-terminal amino acids of a second heterologous G protein subunit as recited in claims 53 and 59.

Moreover, the Examiner acknowledges that Pausch fails to describe each and every limitation of Applicants' claimed invention. At page 20, lines 5-7, the Examiner states, "Pausch et al do not specifically teach substituting the lower limit of C-terminal amino acids (e.g. four amino acids) of GPA1 or substitution of the last five (5) C-terminal GPA1 amino acids . . ." Because each and every limitation of the claims is not found in Pausch, the anticipation rejection of claims 1, and claims 121 and 122, which depend from claim 1, the rejection is improper and should be withdrawn.

Furthermore the Examiner states that Fowlkes describes chimeric G protein subunits where "10, 20, or 40 of the yeast's C-terminal amino acids are substituted." This is not sufficient to

support an anticipation rejection over Fowlkes. Applicants' claims recite a Gpa1 subunit having a C-terminus linked to a heterologous C terminus of another G protein subunit and an N-terminus where at least four amino acids are replaced by four amino acids from a heterologous G protein subunit (claim 1); and a Gpa1 subunit where the last four C-terminal amino acids are replaced with at least the last four C-terminal amino acids of a first heterologous G protein subunit, and in which at least the first five N-terminal amino acids of Gpa1 are replaced with at least the first five N-terminal amino acids of a second heterologous G protein subunit (claims 53 and 59). Fowlkes fails to teach the recited subunits, and therefore cannot destroy the novelty of Applicants' claimed invention. Accordingly, the rejection of claims 1, 53, and 59, and claims 121 and 122, which depend from claims 1 and 53, should be withdrawn.

Claim Rejections - 35 U.S.C. § 103

Claims 1, 53, 57, 59, 121 and 122 are rejected under 35 U.S.C. 103(a) as obvious. In particular:

(1) claims 1, 57, 59, 121, and 122 are rejected over Pausch and Conklin (*Molec. Pharm.* 50:885-890, 2004; hereinafter "Conklin");

(2) claims 1, 53, 57, 59 and 120-122 are further rejected over Pausch, Conklin, and Fowlkes;

(3) claims 1, 53, 57, 59 and 120-122 are further rejected over Fowlkes and Conklin;

(4) claims 1, 53, 54, 57, 59, 60, and 120-122 are further rejected over Pausch, Conklin, and Fowlkes in view of Hamm (*J. Biol. Chem* 273:669-672, 1998; hereinafter "Hamm"). Applicants respectfully disagree.

Applicants note that the rejections of claims 1 and 57 as obvious over Pausch, Conklin, Fowlkes, and Brown were originally made in the Office action mailed October 19, 2004; were addressed in the Reply to Office action mailed April 19, 2004, and were withdrawn in the Office action mailed July 8, 2005.

In the present action, the Examiner has withdrawn the rejection of claims 1, 53, 54, 57, 59, and 60 as obvious over Brown (WO 99/14344), Pausch, Conklin, and Fowlkes in view of Hamm. Applicants note that if claims 1, 53, 57, and 59, are non-obvious over the teachings provided by

these five references, it follows that they must be non-obvious over the teachings present within a subset of these references. For this reason alone, the obviousness rejection of claims 1, 53, 57, and 59 over Pausch, Conklin, and Fowlkes in view of Hamm should be withdrawn. Nevertheless, the rejection over each reference is addressed in detail below.

Pausch and Conklin

Claims 1, 57, 59, 121, and 122 are rejected as obvious over Pausch and Conklin. Claim 1 is directed to yeast cells comprising heterologous amino acids at both the C and N termini. Although the Examiner acknowledges that Pausch fails to describe the replacement of amino acids at the C terminus as recited in Applicants' claims, the Examiner asserts that it would be obvious to determine the minimum number of amino acids that should be replaced in the amino terminus in view of the purported teaching by Pausch that such substitutions might be made. Applicants respectfully disagree and traverse the rejection.

Neither Pausch nor Conklin teaches or suggests modifying the N-terminus portion of GPA-1 to operably link at least the first five N-terminal amino acids of a second heterologous G protein as recited in Applicants' claims. The Examiner acknowledges this at page 22, lines 7-10, where the Examiner states:

Conklin et al., throughout the reference, teach mutations of the **carboxyl-terminal mutations of G-protein α subunit**, as discussed supra.

Both **Pausch et al and Conklin et al, do not specifically teach replacement of amino acid residue at the N-terminus of the chimeric G protein subunit**, as recited in clm 53, and an endogenous yeast pheromone receptor protein is not produced in functional form, as recited in clm 120. (office action mailed August 24, 2007, page 22, lines 7-10).

In the absence of such a teaching or suggestion, the obviousness rejection of the claims over Pausch and Conklin should be withdrawn.

Pausch, Conklin, and Fowlkes

Claims 1, 53, 57, 59, and 120-122 are rejected over Pausch and Conklin in view of Fowlkes. As acknowledged by the Examiner, Pausch and Conklin fail to teach or suggest any modification at the N-terminus of a Gpa1 subunit, much less the specific modifications recited in Applicants' claims. The Examiner cites Fowlkes to remedy the deficiencies of Pausch and Conklin; however, this reliance is misplaced because the Office acknowledged that Fowlkes, like Pausch and Conklin, does not teach or suggest a chimeric G proteins, where the N-terminus of Gpa1 is coupled to at least the first five N-terminal amino acids of a second heterologous G protein subunit as recited in claims. In the Office action mailed on July 9, 2005, the Office stated:

> The above reference teaching(s) [Pausch, Fowlkes, Brown, and Conklin] of a chimeric Galpha protein subunit differs from the presently claimed invention (e.g. claims 1, 53, 54, 57, 59, and 50) by failing to additionally modify the N-terminus portion of GPA1 to operably link/substitute "at least the first five N-terminal amino acids (e.g. the 1st 11 N-terminal amino acids: see claims 54 and 60) of a 2nd heterologous G protein subunit. (page 6, line 19, to page 7, line 2).

As acknowledged by the Office, none of the cited references teaches or suggests linking five amino acids at the N-terminus of a heterologous G protein subunit to the N-terminus of GPA1 as recited in rejected claims 1, 53, and 59, from which the remaining claims depend. Accordingly this basis for the obviousness rejection should also be withdrawn.

Pausch, Conklin, Fowlkes, and Hamm

Claims 1, 53, 54, 57, 59, 60, and 120-122 are rejected under 35 U.S.C. 103(a) over Pausch, Conklin, and Fowlkes, in view of Hamm. The Examiner asserts that Hamm teaches that the N-terminus of the alpha G-protein subunit also appears to be involved in promoting heterologous receptor contact or coupling. In particular, the Examiner asserts that Hamm proposes that modifications should be made in the C-terminal and N-terminal regions of Gα subunits. The Examiner states:

> [T]he Hamm reference provides a clear suggestion and guidance as to how and where to modify the prior art chimeric proteins to produce the claimed invention along with evidence suggesting the modification would be successful; and further enabling methodology (recombination; mutagenesis etc.) to achieve such modification is known in the art and further discussed in the primary references.

Applicants respectfully disagree. The portion of Hamm cited by the Examiner does not suggest making any mutation in Gα or any other polypeptide. The Examiner cites page 669 and Figures 1 and 2. Figure 1 of Hamm is a ribbon diagram showing that the Gα subunit disassociates from the other subunits following GTP binding. Figure 2 is a ribbon diagram showing heterotrimeric G protein interactions with rhodopsin and the membrane lipid bilayer. Regarding Figure 2, Hamm states, "The receptor contacts on the heterotrimeric G protein discussed in the text are red and include amino acids 1-23 and 299-350 of Gα." Hamm describes Gα amino acid residues that contact the rhodopsin receptor. This description of residues where contact between Gα and rhodopsin occurs fails to indicate that any mutations should be made at those residues, or if such mutations were made, that they would result in enhanced coupling.

At page 669, Hamm provides a review of G protein structure and the mechanism of activation of G proteins by receptors. Although Hamm acknowledges that the N-terminal region of Gα is "implicated" in receptor contact (page 669, right column, 4th paragraph), Hamm states: On the α subunit, ***the best characterized receptor contact region is at the C terminus.*** The last 7 amino acids of the α subunit are disordered in the heterotrimer crystal structures, and analysis of receptor-binding peptides selected from a combinatorial peptide library shows that ***these 7 residues are the most critical.*** Studies using chimeric Gα subunits confirm that in fact ***the last 5 residues contribute importantly to specificity of receptor G protein interaction.*** Elegant mutagenesis studies have shown that the C terminus of the third intracellular loop of receptors binds to ***this C-terminal region on Gα subunits.*** (p. 669, third paragraph, Emphasis added.)

By emphasizing the importance of the C terminus in mediating receptor contact and specificity, Hamm and the other cited references not only direct the skilled artisan's attention towards the C terminus, they teach away from modifying the N terminus of the protein. In the absence of a suggestion that modified subunits ***should be made***, and if made, that such subunits would function successfully, the Examiner has failed to establish a *prima facie* case of obviousness. Accordingly, the obviousness rejection is improper and should be withdrawn.

Request for Interview

Applicants are concerned about the length of the prosecution history of the instant application and the fact that they are now dealing with the third Examiner (Examiners B. Celsa and then P. Pannaluri were assigned to this application in the past). Therefore, if upon consideration of the amendments and remarks presented herein, Examiner Liu is inclined to issue a final office action, Applicants respectfully request Examiner Liu contact the undersigned to arrange a telephonic interview **before** issuing a final office action.

CONCLUSION

In view of the above remarks, Applicants believes the pending application is in condition for allowance. Accordingly, the Examiner is respectfully requested to pass this application to issue. Should any of the claims not be found to be allowable, Applicants respectfully request the Examiner to telephone Applicants' undersigned representative at the number below so that a telephonic interview may be scheduled. Applicants thank the Examiner in advance for this courtesy.

Dated: February 26, 2007

Respectfully submitted,

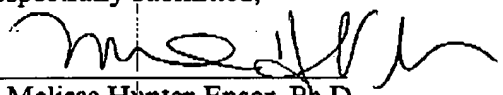
By 

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Exhibit A

Minireview

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 267, No. 1, Issue of January 5, pp. 1–4, 1992

Rhodopsin, Photoreceptor of the Rod Cell

AN EMERGING PATTERN FOR STRUCTURE AND FUNCTION*

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The first steps into the biochemistry of vision were taken by Wald and associates (1), with contributions from Morton and co-workers (2). We owe to them the identification of the chromophore, the nature of its chemical linkage to the visual receptors, the recognition of the light-catalyzed isomerization of 11-*cis*- to all-*trans*-retinal (vitamin A aldehyde), identification of the ensuing photointermediates, and accompanying activation of rhodopsin (1). The chromophore of the visual receptors, in the rods and cones of vertebrate and invertebrate retinae, universally is 11-*cis*-retinal, occasionally dehydroretinal (vitamin A_2), and the primary light transduction step is its isomerization to all-*trans*-retinal. Rhodopsin has received the most attention in studies of its chemistry, biochemistry, and its physiological properties. Indeed, rhodopsin is the best studied of the visual receptors and the superfamily of receptors coupled to GTP-binding proteins (43, 44). A number of reviews on rhodopsin and vision in general have appeared with different emphases (3–10). In this article, chemical and functional aspects of rhodopsin are reviewed first; however, the main motivation is to highlight certain structure-function relationships that are becoming evident within the three structural domains, the intradiscal, the membrane-embedded seven helical, and the cytoplasmic (Fig. 1).

Until recently, the visual photoreceptors were the only systems known to contain the retinal chromophore. However, a second set of retinal-based light-transducing proteins has been found in certain archaebacteria, such as *Halobacterium halobium*. These include bacteriorhodopsin, a proton pump (11), halorhodopsin (12), a chloride pump, and two phototaxis receptors (13). In these four bacterial proteins, light catalyzes all-*trans*- to 13-*cis*-retinal isomerization, and thus, in this group as in all the visual receptors, nature couples specific geometrical isomerizations in the vitamin A polyene system to cycles of conformational changes in the proteins.

Chemical Aspects of Rhodopsin Structure

Opsin genes from a number of sources have been sequenced; the example chosen here is that of bovine opsin. It contains a single polypeptide chain of 348 amino acids whose sequence is known both from protein and DNA sequencing (14–16). One plausible secondary structure model for rhodopsin is shown in Fig. 1. The membrane-water boundaries of the seven membrane-spanning helices are somewhat arbitrary at present. The membrane domain accounts for about 50% of the total protein while the other half is distributed about equally between the cytoplasmic and the intradiscal domains. 11-*cis*-Retinal is linked as a protonated Schiff base to the ε-amino group of Lys-296 in helix G (Fig. 1). The Schiff base, which is protonated in the ground state, is stabilized by a counterion which has been shown to be the carboxylate group of Glu-113 (17–20). The profound influence of this group on the properties of rhodopsin has been clearly established (17, 19). The interaction between Glu-113 and the Schiff base at Lys-296 (Fig. 1) requires that the seven helices in rhodopsin fold to bring helix C and helix G in apposition. (In bacteriorhodopsin, the protonated Schiff base at Lys-216 (helix G) and Asp-85, the probable counterion (helix C), are within 4–4.5 Å of each other.) The retinal-binding pocket in the membrane-embedded helical domain of rhodopsin must contain a large number of amino acids, perhaps about 20, that interact with retinal. From mutagenesis (21) and photoaffinity cross-linking data (22), seven amino acids have been concluded to be proximal to retinal. These are: Phe-115, Ala-117, Glu-122, Trp-126, Ser-127, Trp-265, and Tyr-268. Four of these are included in Fig. 1. Knowledge of the amino acids interacting with retinal is important to the problem of how the signal from retinal isomerization is transmitted to the cytoplasmic domain.

There are 10 cysteines in rhodopsin; however, there is only one disulfide bond, between Cys-110 and Cys-187 (Fig. 1) (23). Cys-322 and Cys-323 carry palmitoyl groups (24) which are probably anchored in the membrane (Fig. 1). Cys-140 and Cys-316 are reactive with sulfhydryl reagents in the dark and are probably exposed to the cytoplasmic surface (25). The reactivities of Cys-167, Cys-222, and Cys-264, which are presumably embedded in the membrane, depend on the reagents and conditions used (26).

Biochemical Aspects of Sensory Functions

Signal Amplification via Transducin (G_t)[1] and cGMP-Phosphodiesterase Cascade—The visual system has evolved to be extremely sensitive to light, such that a single photon is sufficient to excite the rod cell. The chemical and biochemical events that achieve the enormous amplification of the light signal are as follows. Absorption of a photon leads to the isomerization of the C-11, C-12 *cis*-double bond in retinal (Fig. 2) (1). This event initiates the formation of a number of photointermediates from rhodopsin with spectral characteristics and reaction constants shown in Fig. 3. *Meta* II rhodopsin (MII) which contains an unprotonated Schiff base of all-*trans*-retinal is the key intermediate (27) in amplification according to the cascade shown in Fig. 4 (3, 9, 27). Thus MII reacts with G_t-GDP (*step 1*) to form a complex that exchanges GDP for GTP (*step 2*). The resulting complex collapses to $G_t(\alpha)\cdot$GTP and $G_t(\beta\gamma)$, and MII (R*) is free to take another G_t-GDP molecule through GDP-GTP exchange (*step 4*). R* may activate hundreds of G_t-GDP molecules during its lifetime. In *step 5*, $G_t(\alpha)$GTP activates an otherwise inactive cGMP-phosphodiesterase by removing the inhibitory γ subunit, and the latter hydrolyzes cGMP to 5′-GMP (*step 6*).

* The work in my laboratory has been supported by grants GM 28289 and AI 11479 from the National Institutes of Health and from the Office of Naval Research, Department of the Navy N00014-82-K-0189.

[1] The abbreviations used are: G_t, transducin; MII, *meta* II rhodopsin; GTP-binding proteins, G-coupled receptors.

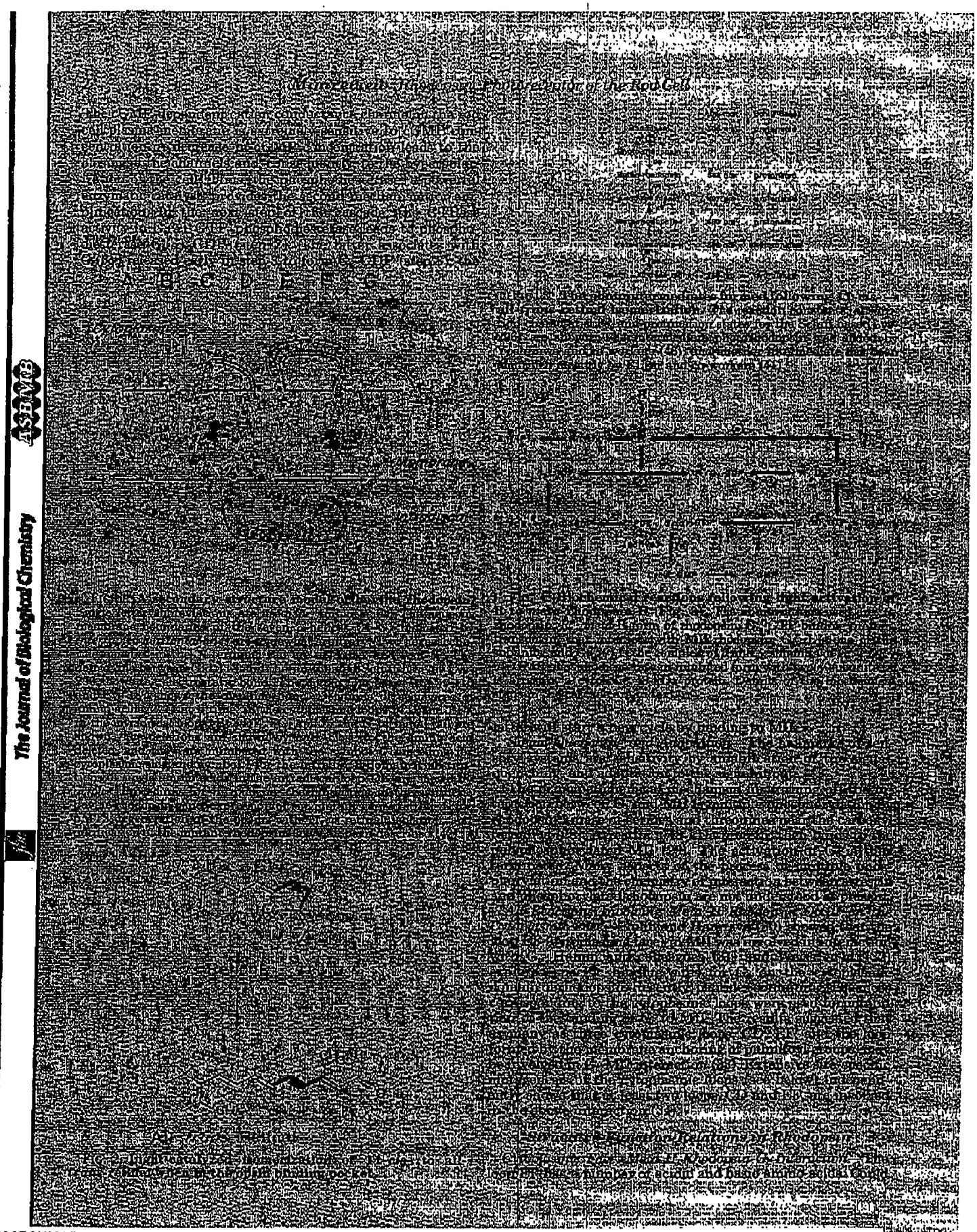

TABLE I

Mutants with amino acid deletions and substitutions in the intradiscal domain

Phenotype I		Phenotype II		Phenotype III	
Mutant designation	Amino acid(s) substituted or deleted	Mutant designation	Amino acid(s) deleted	Mutant designation	Amino acids deleted or substituted
I-18	193–194	I-5	101–103	I-1	7–10
I-19	195–196	I-6	171–172	I-2	11–14
I-20	195–198	I-7	173–174	I-3	18–20
I-21	197–198	I-8	175–176	I-4	21–29
I-22	199–200	I-9	177–178	I-14	183–184
I-23	201–202	I-10	177–182	D190A	190
Y191L	191	I-11	179–180		
Y192L	192	I-12	181–182		
T193V	193	I-13	181–197		
P194A	194	I-15	189–190		
P194G	194	I-16	189–198		
		I-17	191–192		
		I-24	203–204		
		I-25	205–206		
		I-26	207–208		
		I-27	280–283		

they be involved in electrostatic interactions with G_t? This has been tested by a variety of single and multiple amino acid substitutions, for example E239Q, K245L, K245L/K248L, and E247Q/K248L/E249Q. The mutations often showed significant but not critical effects on G_t activation. Thus, there appear to be no critical electrostatic interactions between the loop EF and G_t. In contrast, the charge pair, E134/R135 (helix C), which is conserved in all the G-coupled receptors, is clearly required for binding of G_t to *meta* II rhodopsin. Thus, reversal of the charge pair abolished G_t binding and, in general, substitutions of the charged amino acids by neutral amino acids seriously affected G_t activation (34).

Of the deletions in loop CD (Fig. 1), C-1, a deletion of 8 amino acids, allowed binding of 11-*cis*-retinal, but did not activate transducin. The deletion C-2 failed to bind retinal. However, on replacement of the 13 deleted amino acids in C-2 with an unrelated sequence of the same length (N-terminal residues 2–14 in rhodopsin, Fig. 1), the mutant bound retinal and G_t but did not activate the latter.

Of the five deletion mutations in loop EF (Fig. 1), C-4 containing a four-amino acid deletion showed about 50% of the normal activation of G_t while none of the other mutations (C-3, C-5 to C-7) showed any activity. The results suggest that the C-3 part of the EF loop, closer to the C terminus, is more important for interaction with transducin than is the C-4 region. The mutant C-6 bound G_t but showed no GTPase activity (Franke *et al.*, unpublished work). Thus the most significant conclusions from mutagenesis regarding MII-G_t interaction are: 1) most of the deletion mutants, except those that may not allow proper packing of the helices, bind 11-*cis*-retinal; 2) the MII intermediates formed from these mutants may bind G_t, but they may not allow G_t activation (34); and 3) CD and EF loops are both required for interaction between MII and G_t.

Deletion Mutagenesis Indicates a Structural Role for Intradiscal Domain—Single deletions carried out in the N-terminal tail and in loops BC, DE, and FG (Fig. 1) showed three groups of phenotypes (Table I) (35). Phenotype I resembled wild type rhodopsin, and it was restricted to deletions and amino acid substitutions in the non-conserved part of the loop DE, proximal to helix E (7, 10). Phenotype II (Table I, Fig. 1) did not regenerate rhodopsin-like chromophore, and the glycosylation pattern was markedly different. Phenotype III was observed mainly with the deletion mutants in the N-terminal tail (Table I). These mutants regenerated the rhodopsin chromophore only very poorly, and their glycosylation pattern was different from those of types I and II. When expressed in COS-1 cells, the majority of the mutants (types II and III) described above remained in endoplasmic reticulum whereas the wild type opsin was in the plasma membrane (35). This is, presumably, because the mutant proteins are structurally defective. The fact that rhodopsin mutants, types II and III, can arise by a single relatively short deletion in any one of the loops, BC, DE, or FG, or the N-terminal tail (Table I) suggests that these intradiscal peptide segments cooperatively form a folded structure on the intradiscal face and that this structure is stabilized by the disulfide bond between Cys-110 and Cys-187 (23). Previously, these two cysteines were shown to be required for the formation of functional rhodopsin (36). The deletion mutants presumably cannot form the tertiary structure and, therefore, the disulfide bond. One mutation (C187R) which may result in the lack of the disulfide bond formation in a color vision pigment has been shown to cause the loss of red and green sensitivity (37).

Natural Rhodopsin Mutations in Retinitis Pigmentosa—Recently, there has been a surge of information on mutations in rhodopsin in retinitis pigmentosa patients. More than 30 mutations in intradiscal, helical, and cytoplasmic domains have been identified, and this number is likely to grow. The mutations involve mostly single amino acid substitutions (38–40), often being substitutions by charged amino acids, *e.g.* G101R, G106R, E181K, G188R, P53R, T58R, V87D, G89D, L125R, and C187R. The latter substitutions, especially those that involve insertion of charged groups, are likely to be folding mutations. Examples of such mutations are known from *in vitro* mutagenesis. For example, in rhodopsin the mutants E113K (17) and H211R (22) are defective in folding, and in bacteriorhodopsin the mutant D85H does not undergo folding at acidic pH values, when His is protonated.[2]

The retinitis pigmentosa mutations lend themselves to study by the methods developed for mutagenesis, expression, and chromophore regeneration (42), and some have been expressed in COS-1 cells[3] and in 293S cells (39). Like the intradiscal mutants (Fig. 1), the proteins from these mutants do not move to the plasma membrane, and thus they have some structural defect. Methods need to be developed to study

[2] S. Subramaniam and H. G. Khorana, unpublished observations.
[3] S. Kaushal, unpublished work.

these defects. Is the retinal binding pocket formed? Is the Cys-110/Cys-187 disulfide bond formed? How are the structures of the cytoplasmic and other domains affected? The retinitis pigmentosa mutations seem to open a highly promising new door for studies of rhodopsin structure and function, and by analogy, other seven-transmembrane helical receptors.

Present Status and Work Ahead

While the questions of rhodopsin structure and function are far from being answered, there have been important clues that provide a framework for further investigation. Mutations, site-directed and natural (retinitis pigmentosa), strongly support the notion that the cytoplasmic, transmembrane, and the intradiscal domains each have specific tertiary structures. We assume that the assembly of the molecule begins with the entry of the nascent rhodopsin polypeptide chain into the endoplasmic lumin. Following high mannose glycosylation, the molecule folds, and this includes the formation and insertion of the seven helices into the membrane although their insertion is not structurally coordinated.[4] We further postulate that in the next step, an intradiscal tertiary structure that includes a disulfide bond between C-110 and C-187 is formed. This structure leads to the alignment of the even helices (43) in the membrane and establishment of maximal helix-helix interactions. This results in the formation of the retinal binding pocket[4] and, concomitantly, in the formation of a specific tertiary structure for the cytoplasmic domain. The latter would represent the structure for the "dark" state for rhodopsin that would involve all the loops (AB, CD, EF) as well as the C-terminal tail. Thus, the overall structure embodies the intradiscal domain with a unique structural function, the membrane-embedded helical structure with the critical function of light signal transmission to the cytoplasmic face and the cytoplasmic face that receives the signal and carries out all the biochemistry.

In addition to the host of structural questions that need to be answered, the most prominent unknown is the mechanism of the chemical transmission of the signal following 11-*cis*- to all-*trans*-retinal isomerization to the cytoplasmic face. All the seven helices in the membrane must interact with retinal during the *cis* → *trans* isomerization. It is clear that as a consequence of the tertiary structure comprising the intradiscal domain, the perturbation following *cis* → *trans* isomerization will be transmitted specifically to the cytoplasmic face. What is the chemical nature of the transmission by the helices? Will the helices rotate, move laterally, or move up and down? Understanding the mechanism of the coupling between retinal and the protein and the accompanying structural changes in the protein is clearly at the heart of the visual transduction problem.

It is reasonable to expect that the structure-function pattern presented above for rhodopsin will apply to all the visual pigments as well as to a majority of the G-coupled receptors. The conserved sequences in different regions of the receptors (7, 10, 44, 45) support this conclusion. Further, like rhodopsin, 7-helix receptors should have disulfide bonds in functionally equivalent positions. Visual pigments and most of the G-coupled receptors (44, 45), in fact, have cysteine residues in matching places (helix C and loop DE) that can potentially form disulfide bonds like the C-110/C-187 bond in rhodopsin.

Acknowledgments—I wish to warmly acknowledge the contributions, both theoretical and practical, of former colleagues (Tomoko Doi, Roland Franke, Sadu Karnik, Barry Knox, Tomoko Nakayama, Dan Oprian, Tom Sakmar, and Debra Thompson) and present colleagues (Florence Davidson, Shalesh Kaushal, John Resek, and Kevin Ridge).

[4] This is suggested by the fact that the defective intradiscal mutants that stayed in endoplasmic reticulum did not bind 11-*cis*-retinal. The seven helical segments were thus not aligned. We believe that the information for the alignment of the helices does not come from the insertion of the helices into the membrane but from the formation of the tertiary structure including the disulfide bond (*cf.* Ref. 43).

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Exhibit B

Minireview

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 267, No. 10, Issue of April 5, pp. 6451–6454, 1992

Adenosine Receptors

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The physiological consequences of adenosine and adenine nucleotides have been recognized for more than 60 years (1, 2). What makes these substances unusual is that they may produce their effects both by intracellular and extracellular mechanisms (3). Both adenosine and adenine nucleotides are found in all living cells as part of the normal metabolic machinery that runs the cells, and their concentrations are dynamically regulated by a variety of pathophysiological conditions (2, 3). In addition, under appropriate conditions these compounds can be released from cells where they can interact with specific cell surface receptors to modulate cellular function in an autocrine or paracrine manner (3, 4). The focus of this review is on adenosine and adenosine receptors, and the readers are referred to recent reviews for information on adenine nucleotides and their receptors (5, 6). Adenosine is produced intracellularly via two distinct metabolic pathways, both of which involve hydrolases (3). The first involves the hydrolysis of AMP to adenosine by 5′-nucleotidase and the second the catabolism of *S*-adenosylhomocysteine (3). Under conditions of stress, such as hypoxia, when the cellular energy state is depressed, intracellular adenosine levels acutely increase and adenosine is released from the cell (3). This released adenosine can then act on adenosine receptors as a local metabolic regulator or as Newby has termed it, a "retaliatory metabolite" (7). Activation of the receptor then alters the activity of a second messenger system in an attempt to bring the tissue or cell back into a normal equilibrium or to protect it from overactivation. The short half-life of adenosine in the circulation makes it highly unlikely that adenosine acts as a circulating hormone (3). Adenosine once released from the cell has several pathways by which it can be eliminated. First, it can be transported back into the cell by a specific nucleoside transporter which has been characterized as a 60-kDa protein (8). Adenosine can also cross the cell membrane by simple diffusion. Finally, adenosine can be deaminated to the receptor-inactive inosine by adenosine deaminase (3).

Adenosine once released can activate adenosine receptors which in turn regulate a diverse set of physiological functions. These include cardiac rate and contractility, smooth muscle tone, sedation, release of neurotransmitters, platelet function, lipolysis, renal function, and white blood cell function (3, 9). Thus, almost all organ systems of an animal are regulated by the local release of adenosine (9). A major question in adenosine research is how adenosine produces these diverse physiological effects and what second messenger systems are responsible. To begin to understand these complex physiological responses it is necessary to probe the receptors and signaling apparatus involved in transducing the signal across the cell membrane.

Receptor Subtypes

Purinoceptors were originally classified by Burnstock (10) as P_1 and P_2 purinergic receptors depending on their preference for adenosine or adenine nucleotides. The relative potencies for P_1 sites are adenosine > AMP ≥ ADP ≥ ATP, while potencies for P_2 sites are ATP ≥ ADP ≥ AMP ≥ adenosine. P_1 and P_2 sites can also be distinguished by the insensitivity of P_2 sites to antagonism by methylxanthines which are effective antagonists at P_1 sites. The adenosine-sensitive P_1 sites are the adenosine receptors, which have now been further subdivided into A_1 and A_2 subtypes on the basis of their differential selectivity for adenosine analogs (11). The potency series for A_1 adenosine receptors (A_1AR)[1] is *R*-phenylisopropyladenosine (*R*-PIA) > 5′-*N*-ethyl-carboxamide adenosine > *S*-PIA, while the A_2AR potency series is 5′-*N*-ethylcarboxamide adenosine > *R*-PIA > *S*-PIA (11). Recently a number of A_1AR-selective antagonists have been developed that are useful in characterizing and differentiating A_1AR from A_2ARs (3, 12).

Further division of A_1ARs and A_2ARs into subclasses has been proposed based on a variety of pharmacological criteria, and although these are not universally accepted, the recent cloning of the adenosine receptor (see below) will likely lead to a proliferation of subclasses in the near future (13).

Receptor Structure

A_1AR structure has been studied at three separate levels. The A_1AR has been covalently labeled using ^{125}I-labeled agonist and antagonist photoaffinity and affinity probes in a wide variety of tissues (14–16). This approach demonstrates that the A_1AR migrates as a M_r 36,000 protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (14–16). The labeled protein displays all of the pharmacologic properties expected of the A_1AR. Proteinase sensitivity studies suggest that agonists when binding to the A_1AR induced a unique conformational change in the receptor compared with antagonists (17). This finding supports the notion that it is the unique ability of agonists to promote a transmembrane signal by altering the receptor's conformation, thus permitting it to functionally activate multiple G proteins. This activation leads to modulation of the appropriate effector system. In contrast, antagonists simply occupy the receptor and do not induce the needed conformational change to activate G proteins.

Purification (25,000-fold) of the A_1AR to apparent homogeneity through a series of chromatographic steps has now been accomplished by a number of groups (18–20). The purified A_1AR receptor migrates as a M_r 36,000 protein and retains all the appropriate binding characteristics. The single purified peptide is the fully functional receptor in that it can bind ligands with appropriate pharmacology and functionally activate G proteins (20). Further studies have documented that A_1ARs are glycoproteins that contain a single complex type

‡ Supported in part by National Heart, Lung, and Blood Institute Grant RO1-HL35134, Grant-in-aid 91006200 from the American Heart Association, and Specialized Center of Research Grant P50HL17670.

[1] The abbreviations used are: AR, adenosine receptor(s); PIA, phenylisopropyladenosine; G_i, inhibitory guanine nucleotide binding protein(s); G_s, stimulatory guanine nucleotide binding protein(s).

carbohydrate chain (15, 19). The functional role of the glycan component of the receptor remains enigmatic.

Recently, a number of A_1ARs have been cloned, sequenced, and expressed (21–24). These include the A_1ARs from dog thyroid, rat brain, and the bovine brain. All have been cloned from cDNA libraries (21–24). These receptors are small (M_r ~ 36,500) compared with some G protein-coupled receptors but fit well into the seven-transmembrane domain spanning motif (21). Although the A_1ARs from dog and rat share similar pharmacological properties, the A_1AR from bovine brain has a unique pharmacological profile and distinct affinities for agonists and antagonists (19, 24). The bovine brain A_1AR is, therefore, likely a subclass of the family of A_1ARs. Shown in Fig. 1 is a model of the A_1AR. The A_1AR has several unusual properties compared with other G protein-coupled receptors including: 1) one or two consensus glycosylation sites on the second extracellular loop rather than site(s) on the amino terminus; 2) a very short third intracellular loop (34 amino acids) compared with other receptors that inhibit adenylate cyclase, which typically have 140–160 amino acids in the third intracellular loop; 3) a relatively low molecular weight of 36,500 with 326 amino acids; 4) a potential site for fatty acylation on the carboxyl tail; and 5) very few consensus sequences for phosphorylation (24). This latter finding may have direct functional consequences since it has recently been appreciated that inhibitory receptors desensitize much more slowly than stimulatory receptors (see below). All three of the cloned A_1AR have been expressed in a variety of cells and shown to inhibit adenylate cyclase (21–24).

The A_2AR from dog thyroid has also been cloned and expressed (22, 25). The A_1AR and A_2AR have many structural similarities and share a 51% identity at the amino acid level within the transmembrane domains (22). As predicted from photoaffinity labeling experiments, the A_2AR is of greater mass (44,971) than the A_1AR (26, 27). The A_2AR has a greater number of potential phosphorylation sites than the A_1AR and is regulated much more rapidly than an A_1AR (28). The A_3AR also has its glycosylation site in the second extracellular loop (22). In contrast to the A_1AR it has a fairly normal sized third cytoplasmic loop for stimulatory receptors and does not contain a site for fatty acid acylation (22).

Receptors Effector Coupling

Originally, A_1AR and A_2AR were known to inhibit and stimulate adenylylcyclase, respectively (11). A_2ARs are still thought to couple unifocally to adenylylcyclase. In contrast, A_1ARs are now known to couple to a variety of effector systems (3, 29) (see Table I). The most comprehensively studied effector is, of course, adenylylcyclase, but it is well documented that A_1AR acting through pertussis toxin-sensitive G_i proteins can open K^+ channels (outward rectifying) in cardiac tissue. Many of the adenosine-mediated electrophysiological effects used for therapeutic benefit appear related to alterations in K^+ conductance rather than alterations in cAMP (30).

A_1AR appears to modulate phospholipase C activity, but the exact mechaniams remain enigmatic (3). In some systems the A_1AR's effect appears to be indirect in that it only modulates phospholipase C activity after another egonist, such as histamine, has activated the enzyme (3). In addition, A_1AR can, depending upon the species studied, either inhibit or stimulate phosphoinositol turnover in brain slices (3). Much additional work needs to be performed before any true understanding of the role of adenosine receptors in regulating phospholipase C can be stated conclusively.

There is an abundance of information on the ability of A_1ARs to inhibit Ca^{2+} channel opening in neurons (3). One mechanism for this inhibition is likely related to hyperpolarization of the membrane following increases in K^+ conductance. A second distinct mechanism for inhibiting the Ca^{2+} channel opening is the activation of a G protein which can directly inhibit the channel protein (3). No precise mechanism for this action is at present known.

Studies in 1987 suggested that A_1AR could activate guanyl-

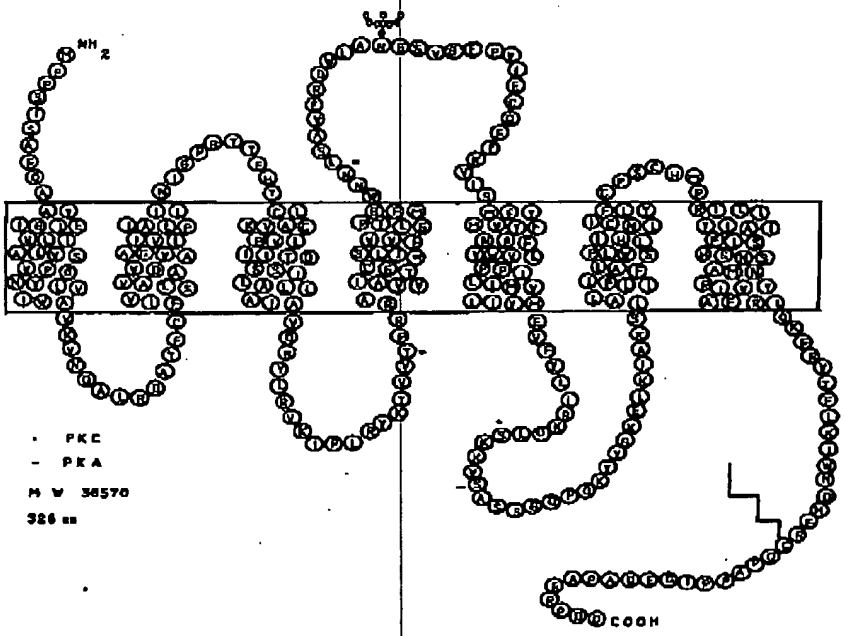

FIG. 1. Shown is the amino acid (*aa*) sequence of the A_1AR in a format which portrays the receptor with a seven-transmembrane domain motif. This is the general model for all G protein-coupled receptors. The amino terminus is extracellular. The consensus sequences for glycosylation are on the second extracellular loop. A single carbohydrate chain is shown, and the other potential site is marked by the *asterisk*. The potential site for fatty acid acylation is shown by the *zigzag line* on the carboxyl tail. Potential sites for phosphorylation by protein kinase A (*PKA*) and protein kinase C (*PKC*) are shown.

TABLE I

Adenosine receptor—G protein effector coupling

$rG_{i\alpha3}$, the recombinant form of the α subunit of the inhibitory G protein type 3; PLC, phospholipase C; PLA_2, phospholipase A_2.

Receptor	G protein interaction	Effector
A_1AR	$rG_{i\alpha3} > rG_{i\alpha2} = rG_{i\alpha1} \cong rG_{o\alpha}$ $rG_{s\alpha}$ and $rG_{z\alpha} \rightarrow$ no coupling	Adenylylcyclase ↓ K^+ channel ↑ Ca^{2+} channel ↓ PLC ↑,↓ PLA_2 ↓ Glucose transporter ↑
A_2AR	G_s	Adenylylcyclase ↑

ate cyclase in smooth muscle cells (31). No subsequent studies have appeared, making further analysis of this putative receptor-effector coupling difficult. A variety of other effector systems have been reported to be coupled to A_1AR, and these include phospholipase A_2, the glucose transporter, a low K_m phosphodiesterase, Cl^- transport, and Na^+/Ca^{2+} exchange (3).

A major question for all receptors that couple to G proteins is how selectively does a specific receptor couple to the G proteins in a membrane. This question is now being addressed using purified components. Very recent reconstitution studies using purified A_1AR and recombinant G protein α subunits have demonstrated that the A_1AR couples with highest affinity to α_{i3} with α_{i2} and α_{i1} having ~10-fold lower affinity. Recombinant α_o also functionally couples to A_1AR while α_s and α_z do not (20). Which of these α subunits functionally interacts with the A_1AR in a specific cell and then couples to an effector system remains speculative. What is clear is that the A_1AR can couple to a variety of G proteins and effector systems, and where the selectivity and specificity reside needs to be determined.

Receptor Regulation

Regulation of transmembrane signaling systems permits a cell to protect itself from either over- or underactivation of an effector system. The most commonly studied regulatory process for G protein-coupled receptors has been agonist-induced desensitization (29, 32). Although much is currently known concerning desensitization of receptors that stimulate adenylylcyclase, much less is known about inhibitory receptors or receptors, which couple to effector systems other than adenylylcyclase such as K^+ channels.

A general theme that has developed is that persistent agonist activation leads to an uncoupling of a receptor from its effector system. This uncoupling likely involves multiple different mechanisms which can occur singly or in combination. These include alterations in the quantity of the receptor by down-regulation or sequestration, regulation of receptor transcription and/or translation rates, alteration in the quantity of the G protein themselves, and covalent modification of the receptor such as by phosphorylation (29, 32). Phosphorylation likely uncouples the receptor from its G protein. Which of these mechanisms is utilized depends on the cell type studied, the concentration of agonist present, and the duration of agonist exposure.

It is now clear that desensitization of the inhibitory A_1AR proceeds at a slow rate compared with stimulatory receptors in that the process takes hours to days rather than minutes to hours. This has been found in both *in vivo* models as well as in cell culture models (28, 33, 34).

An *in vivo* rat adipocyte model in which *R*-PIA was infused for periods up to 6 days was first described (33, 34). Adipocytes have both A_1AR and prostaglandin E_1 receptors that inhibit adenylylcyclase. After *R*-PIA treatment, the ability of both *R*-PIA and prostaglandin E_1 to inhibit adenylylcyclase is diminished by 50%. This process took 4–6 days to occur. Surprisingly, it was found that stimulatory agents such as isoproterenol (acting via β-adrenergic receptors), sodium fluoride (acting via G_s), and forskolin (acting via the catalytic unit) all demonstrated an enhanced ability to generate cAMP (33). Interestingly, the enhanced production of cAMP occurred by 2 days, long before desensitization of A_1AR became apparent. Analysis of the transmembrane signaling apparatus of the cell membrane revealed that β-adrenergic receptors were unchanged, while A_1ARs were down-regulated and partially uncoupled from G_i (33, 34). Quantitation of the G proteins revealed that by 2 days of treatment, G_s was significantly increased (~40%) while G_i was not altered. By day 4, G_s was increased by ~80% with only a small decrease in G_i. There was no further increase in G_s at day 6, but G_i proteins (actually α_{i1} and α_{i2}) were decreased by 60%. These changes occurred without any concomitant changes in the mRNAs for α subunits (34). These data suggest that regulation of the G proteins may occur at the protein stability level. Several important points were gleaned from this *in vivo* model. First, a heterologous pattern of desensitization occurs; second, multiple components of the system are regulated over a long period of time (6 days); third, a sensitization to stimulatory agents is readily apparent; and, finally, there is temporal dispersion between the effects on the stimulatory *versus* the inhibitory pathways.

Recent studies in DDT_1 MF-2 smooth muscle cells document that the A_1AR-adenylylcyclase system can be desensitized by *R*-PIA over a 24-h period with a $t_{1/2}$ of ~10 h. In contrast with the *in vivo* model, this desensitization process is associated with a decrease in A_1AR number, an internalization of the A_1AR, and an uncoupling of A_1AR from G_i, but no alterations in the quantity of the G proteins or a sensitization to stimulatory agents occur (28). In addition, we have been able to document that the desensitization of A_1AR is associated with a phosphorylation of A_1AR (28). From cloning studies, it is known that there are consensus sequences for protein kinase A, protein kinase C, and the β-adrenergic receptor kinase. However, it is currently unknown which kinase is responsible for the phosphorylation or if phosphorylation alters receptor function.

The DDT_1 MF-2 cell line also contains the A_3AR (28). Despite the fact that the A_1AR and the A_2AR share the same endogenous ligand and are coexpressed in the same cell, it appears the mechanism(s) by which they desensitize are quite disparate. Using synthetic selective agonists it is possible to desensitize each receptor subtype without effecting the other. Desensitization of the A_2AR is homologous and occurs rapidly (~20 min) with complete desensitization within ~1 h (28). This desensitization is not associated with down-regulation (28). Lack of an antagonist radioligand selective for A_2AR precluded further analysis. Information on the desensitization of A_2AR is only now beginning to appear.

The fact that a single cell type contains both A_1- and A_2AR presents an interesting dilemma in cell biology. It is unclear why two receptors which have opposing effects on adenylylcyclase are coexpressed in the same cell. This coexpression is not unique to these cultured cells but has also been reported in adipocytes. Whether one of these receptors couples to another yet unknown effector system which biochemically predominates over changes in cAMP remains unknown. In addition, the mechanism responsible for tissue-specific expression of each receptor subtype is unknown.

The phenomenon of antagonist-induced sensitization has received much attention. Chronic administration of AR antagonists, such as caffeine or theophylline followed by abrupt

withdrawal, has been associated with a constellation of symptoms known as the "caffeine withdrawal syndrome" (35). Multiple laboratories have now documented that chronic antagonist administration is associated with an increase in A_1AR number, an enhanced coupling of A_1AR with G_i, and an increased quantity of α_i. These changes are associated with an enhanced ability of *R*-PIA to inhibit adenylylcyclase (36, 37). In addition, it has been documented that the number of β-adrenergic receptors is decreased following caffeine treatment (38). All of these modifications would tend to promote a suppression of cAMP in the brain. The sudden fall in cAMP following abrupt withdrawal of the methylxanthines may provide a molecular mechanism responsible for the caffeine withdrawal syndrome and the sensitization to the physiological effect of adenosine. Regulation of adenosine receptors is also known to occur with alterations in thyroid hormone and glucocorticoid levels (39, 40).

Summary

With the recent purification and cloning of the A_1AR and the cloning of the A_2AR in association with the development of selective radioligands, we are now poised to begin to understand at the most fundamental level the structure, function, and regulation of adenosine receptors. Although adenosine's physiological effects have been appreciated for more than 60 years, we are only now ready to address questions at the biochemical and molecular biological levels. We are likely to begin to see evidence for a whole group of adenosine receptors undetected by previous technology. One era of adenosine receptor research has just ended, and we now enter the new with anxious anticipation.

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Exhibit C

Diversity of structure, signaling, and regulation within the family of muscarinic cholinergic receptors

ABSTRACT **Acetylcholine signals through two types of unrelated membrane receptors referred to as nicotinic (nAChR) and muscarinic (mAChR) acetylcholine receptors. Nicotinic acetylcholine receptors were the first neurotransmitter receptors to be purified, cloned, and sequenced, and much is known about these proteins. In contrast, until 5 years ago relatively little was known about the muscarinic receptors. Since then there has been an explosion of information concerning the structure, signaling, and regulation of what is now known to be a family of muscarinic receptors. This review discusses the five identified members of the mAChR family and their coupling to the multiple G proteins that allow mAChRs to modulate many different types of signal transduction pathways. The five members of this family that have been identified so far have striking homology in their hydrophobic membrane domains but possess distinct cytoplasmic domains between the fifth and sixth membrane-spanning regions. These cytoplasmic domains appear to contain important determinants for receptor/G protein interaction and are likely to contain phosphorylation sites that regulate these interactions. mAChR agonists have been shown to induce phosphorylation of mAChR in intact cells, and the evidence that suggests that receptor phosphorylation may play a role in the regulation of receptor function is discussed.—Hosey, M. M. Diversity of structure, signaling, and regulation within the family of muscarinic cholinergic receptors. *FASEB J.* 6: 845-852; 1992.**

Key Words: receptor phosphorylation · acetylcholine

THE TWO TARGETS OF ACETYLCHOLINE: THE MUSCARINIC AND THE NICOTINIC CHOLINERGIC RECEPTORS

The regulatory effects of the neurotransmitter acetylcholine (ACh[1]) are diverse. Acetylcholine regulates the activity of peripheral excitable cells innervated by the autonomic nervous system, as well as many different types of neurons innervated by cholinergic inputs throughout the central nervous system. When one considers that this single molecule, acetylcholine, is involved in regulating such diverse physiological activities as muscle contraction, heart rate, secretion, and memory retention, perhaps it is not so surprising that there is more diversity in the structure, signaling and regulation of cholinergic receptors than was previously recognized. Signal transduction mediated by acetylcholine occurs through two very different classes of receptors that are known as the muscarinic and nicotinic cholinergic receptors. These receptors were originally recognized as being distinct in that they could be differentially activated by nicotine and muscarine. The marvelous tools of molecular biology have now revealed that there are really two, not-so-small *families* of cholinergic receptors, rather than simply two pharmacologically distinct binding proteins.

MUSCARINIC AND NICOTINIC RECEPTORS ARE STRUCTURALLY UNRELATED PROTEINS

The first striking feature about the two types of acetylcholine receptors is that they are structurally unrelated molecules that are members of different receptor superfamilies. Nicotinic receptors (nAChR) belong to the superfamily of receptor-gated ion channels, whereas muscarinic receptors belong to the superfamily of G protein (GTP binding regulatory proteins)-coupled receptors (1). The nicotinic receptors are pentameric membrane proteins that form nonselective cation channels (1). These proteins consist of two to four distinct hydrophobic subunits that share a high degree of homology. Different combinations of subunits, including $\alpha_2\beta\gamma\delta$, $\alpha_2\beta\delta\varepsilon$, $\alpha_2\beta_3$, have been observed in different tissues and/or at different developmental stages (1). Acetylcholine binds to the α subunits of the nAChR, resulting in opening of the ion channels. In marked contrast, muscarinic receptors (mAChR) are single subunit proteins that are predicted to have seven transmembrane-spanning regions and a fairly large cytoplasmic domain between the fifth and sixth membrane-spanning regions (see Fig. 1). Binding of acetylcholine to the mAChR may involve various hydrophobic domains (2) and likely causes conformational changes in the cytoplasmic regions that couple to G proteins.

Thus, the two main types of acetylcholine receptors do not share the same lineage. This is unusual with signaling molecules. Other neurotransmitters, hormones, growth factors, and ligands may act through multiple forms of related receptors, but it is unusual for regulatory ligands to act through totally unrelated receptor molecules. It is remarkable that the muscarinic type of cholinergic receptors are more homologous to the receptors for norepinephrine, dopamine, and light than they are to the nicotinic receptors, which in turn are more homologous to receptors for glutamate and glycine than to muscarinic receptors. As more details are revealed about the binding domains for acetylcholine in the nicotinic and muscarinic cholinergic receptors, it will be interesting to know what commonalities will be found in these sites. The pharmacology for the two receptors is

[1]Abbreviations: ACh, acetylcholine; mAChR, muscarinic cholinergic receptor; nAChR, nicotinic acetylcholine receptor; PI, polyphosphoinositide; βARK, β-adrenergic receptor kinase; PKC, protein kinase C; PKA, cAMP-dependent protein kinase; G proteins, GTP binding regulatory proteins.

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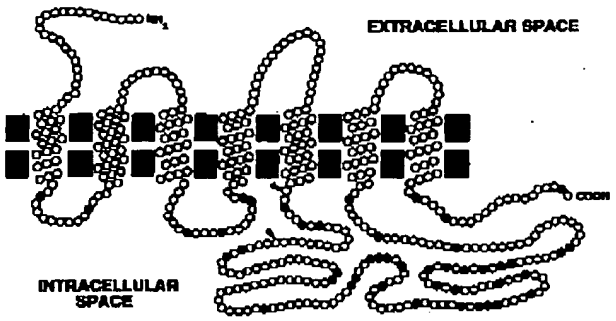

Figure 1. Putative structure of a muscarinic receptor. This generic representation of mAChR subtypes is based on the human m2 mAChR (10). The mAChR subtypes are highly homologous in the membrane-spanning regions but exhibit considerable sequence diversity in the third cytoplasmic loop. The area in the NH_2-terminal region of the third cytoplasmic domain that has been found to impart selectivity in m2/G protein interaction (37) is indicated by the arrowheads. Cytoplasmically located serines and threonines in the m2 mAChR that could potentially be phosphorylated are represented by the solid circles and stippled circles, respectively. The only cytoplasmically oriented serines in all mAChR subtypes are in the third cytoplasmic loops (10–13).

strikingly different (with a few exceptions); nevertheless, the physiological regulator, acetylcholine, is the same.

FAMILIES OF NICOTINIC AND MUSCARINIC RECEPTORS

The most recent revelation about the cholinergic receptors is that there are fairly large families of nicotinic and muscarinic receptors. All members of the nicotinic receptor family are multisubunit proteins. Elegant studies have addressed the roles of the various subunits and much has been learned about the structural basis underlying the functional diversity of the members of this superfamily of receptors (for example, see ref. 1). Much has been written about the nicotinic receptors, as these were the first neurotransmitter receptors to be purified, reconstituted, cloned, and expressed. In fact, work on this type of receptor so dominated the field of cholinergic receptors that for a while many investigators who worked with nicotinic receptors often referred to these as "the" acetylcholine receptors, and the muscarinic receptors seemed to be poor relatives that were lost in the excitement. Tables turn! Recently, the field of muscarinic receptors has had its share of glittering success. Thus, the focus of the remainder of this short review will center on the molecular aspects of the historically less well appreciated, but certainly not less interesting, family of muscarinic cholinergic receptors.

MOLECULAR DIVERSITY OF MUSCARINIC CHOLINERGIC RECEPTORS

Pharmacological studies first suggested that more than one type of muscarinic cholinergic receptor might exist. A key finding was that the drug pirenzepine was found to act as a high-affinity antagonist at mAChR in certain brain regions, whereas it appeared to have almost a hundred-fold lower affinity for cardiac mAChRs (3). This discovery led to many related investigations, and the consensus conclusions were that the brain contained muscarinic receptors, referred to as M1 receptors, that differed from those in the heart, referred to as M2 receptors, and that receptors in endocrine cells could not be classified as either M1 or M2.

Biochemical investigations into the molecular basis of this pharmacological diversity were aided by the availability of the high-affinity antagonists [^{3}H]quinuclidinyl benzilate (QNB) and [^{3}H]N-methylscopolamine (NMS), the irreversible affinity analog [^{3}H]propylbenzilylcholine mustard, and a highly specific and useful affinity chromatography system, ABT-Sepharose (4). With these tools the muscarinic receptors were identified as 70- to 80-kDa proteins, and no major differences were observed in the molecular sizes of the receptors from different tissues. Subsequently, the receptors from porcine brain and heart were purified (5, 6) and sufficient material was available to obtain limited amino acid sequence data.

In late 1986, Numa's laboratory, which had previously cloned and expressed nictonic cholinergic receptors (1), published the results of the first cDNA cloning and expression of a brain M1 receptor (7). Later that same year Kubo et al. (from Numa's laboratory) published the sequence, from the cDNA, of the porcine heart M2 receptor (8). This was confirmed in early 1987 by a second group that also expressed the heart M2 receptor in mammalian cells and showed that it attenuated cAMP formation (9). The results from these studies demonstrated that the heart M2 and brain M1 muscarinic receptors were related, but different. The amino acid sequences were found to be 38% identical. Both the brain M1 and heart M2 muscarinic receptors were found to possess considerable homology to two previously cloned and sequenced receptors, the light receptor rhodopsin and the β-adrenergic receptor. Thus, the muscarinic receptors were the third identified members of the ever-growing "magnificent seven" family of G protein-coupled receptors, all of which are predicted to possess seven membrane-spanning regions.

It is now recognized that there are not only two, but at least five, subtypes of muscarinic receptors, and that all five identified subtypes are expressed in mammalian brain (10–13). These five mAChR subtypes (now referred to as m1, m2, m3, m4, and m5 according to the nomenclature of Bonner et al., ref 11; see ref 14 for details of nomenclature) have similar predicted membrane topographies, and are homologous primarily in their membrane-spanning domains (Fig. 1). Notably, the major difference among these receptors is that they each possess a unique cytoplasmic domain in the third loop connecting the fifth and sixth transmembrane domains (10–12). Although the cytoplasmic domains for each of the receptor subtypes are very different from each other, where there is similarity in these domains it is more extensive for those receptors that couple to the same signal transduction pathways (i.e., m1, m3, and m5 are more similar to each other than to m2 and m4, and vice versa; see below).

MUSCARINIC RECEPTOR SUBTYPES COUPLE TO A VARIETY OF SIGNALING PATHWAYS

The divergence in the cytoplasmic domains of mAChR subtypes appears to play a major role in accounting for

the diversity in the signal transduction events that are mediated by the mAChR subtypes, and may also be critical in defining how each receptor subtype is regulated. The mAChR subtypes couple to multiple G proteins to modulate many different signal transduction pathways (Fig. 2), including stimulation of phospholipases C, A_2, and D, attenuation of cAMP synthesis, stimulation of cAMP degradation, stimulation of cGMP production, and regulation of several ion channels (15–27). Studies with various cell types that have been transfected with vectors containing the cDNAs for the five identified mAChR subtypes have convincingly demonstrated that the m1, m3, and m5 mAChR subtypes can couple vigorously to stimulation of polyphosphoinositide (PI) hydrolysis (12, 13, 16, 17, 25), whereas the m2 and m4 receptors signal attenuation of adenylyl cyclase, but are only weak stimulators of PI hydrolysis (and apparently this latter event occurs only in some cells) (15–17, 25). Studies of other signaling events initiated by the cloned mAChR subtypes have revealed differences in their abilities to stimulate phospholipases and ion channels (16, 18, 20–27).

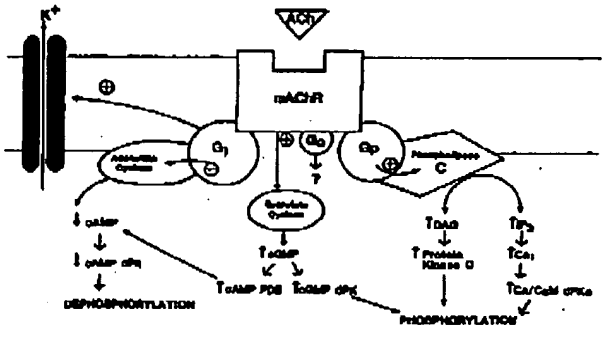

Figure 2. Some of the signal transduction pathways modulated by muscarinic receptors. This schematic representation is based on events regulated by mAChR in cardiac cells, but is generally applicable to other cells types, although not all signaling pathways that are regulated by mAChR subtypes are illustrated. The attenuation of adenylyl cyclase and the stimulation of K^+ channels appear to involve signaling through G_i proteins. The stimulation of PI-specific phospholipase C may involve a G_p (pertussis toxin-insensitive G protein that stimulates phospholipase C) such as the recently described G_q (28) and/or, in certain cells, pertussis toxin-sensitive G proteins such as G_o and G_i. As described in the text, the attenuation of adenylyl cyclase occurs through m2 and m4 mAChR subtypes, whereas the stimulation of phospholipase C occurs most vigorously with m1, m3, and m5 receptors, and to lesser extents with m2 and m4 receptors. The question mark after G_o indicates that the consequences of mAChR/G_o interaction are not well established. The mechanism by which mAChR increase cGMP production appears to occur secondarily to increases in intracellular Ca^{2+} and activation of a Ca^{2+}/calmodulin dependent NO synthase, which in turn leads to activation of soluble guanylate cyclase (32). Increased intracellular Ca^{2+} and/or cGMP may also contribute to decreased cAMP levels via activation of a Ca^{2+}/calmodulin sensitive or cGMP-sensitive phosphodiesterases, respectively.

This diversity in signaling is achieved in several ways. First, it appears that a single mAChR subtype is capable of activating more than one type of G protein in a single cell type. This is illustrated by elegant studies performed with CHO cells transfected with m1 receptor cDNAs where pertussis toxin, which ADP-ribosylates G_i and G_o and prevents receptor/G_i or G_o interactions, was used to differentiate the types of G proteins that interact with the m1 mAChR. In these cells, the m1 receptors stimulate PI hydrolysis through pertussis toxin-sensitive and insensitive G proteins (17). These results also demonstrate that a single type of mAChR can activate more than one G protein to regulate one type of effector (1 receptor/2 G proteins/1 effector).

In addition, a single mAChR is capable of coupling to more than one effector. For example, in CHO cells transfected with the m2 mAChR subtype (15), the m2 receptors attenuate adenylyl cyclase and stimulate PI hydrolysis. The coupling to cyclase is inhibited by pertussis toxin, whereas stimulation of phosphoinositide hydrolysis is much less sensitive to pertussis toxin, suggesting the involvement of another G protein (15). Similarly, in mammalian cardiac cells that contain predominately (or perhaps exclusively) the m2 mAChR, the mAChRs attenuate adenylyl cyclase and activate K^+ channels in a pertussis toxin-sensitive manner, and stimulate PI hydrolysis in a pertussis toxin-insensitive manner (29, 30). Furthermore, cardiac cells contain almost as much G_o as G_i, and cardiac mAChR are also capable of activating G_o (31), although the physiological role of mAChR/G_o coupling in heart remains unknown. Thus, a single type of mAChR appears to couple to one or more G proteins to regulate several signal transduction pathways.

Can the coupling of a single mAChR subtype to multiple effectors occur through one G protein? It has not been definitively demonstrated whether, in the microenvironment of a single cell, a single G protein is used to switch on or off multiple effectors. For example, it is not clear if the cardiac m2 mAChR activate K^+ channels through the same G_i protein it uses to attenuate adenylyl cyclase. Although many isoforms of G_i have been discovered and tested for differences in their functional capabilities, studies with recombinant isoforms of G_i have demonstrated that all forms so far tested can support activation of K^+ channels (30). Many laboratories are now determining if specific isoforms of G_i and G_o are used for the different signaling events regulated by mAChR.

The events involved in the activation of some signal transduction pathways by mAChR subtypes have not been studied as extensively as others. For example, less is known about the receptor-mediated activation of phopholipases A_2 and D (18, 26, 27). The mechanism or mechanisms involved in the muscarinic receptor-dependent activation of cGMP synthesis appears to result secondarily from a Ca^{2+}-dependent step. In this instance a receptor-mediated increase in intracellular Ca^{2+}, such as occurs after receptor-mediated increases in IP_3, is believed to lead to activation of a Ca^{2+}/calmodulin-dependent nitric oxide (NO) synthase, which in turn leads to increased NO formation and activation of soluble guanylate cyclase (for example, see 32). These and other signaling events modulated by mAChR subtypes are the subjects of ongoing investigations.

THE THIRD CYTOPLASMIC LOOPS CONTAIN MAJOR DETERMINANTS FOR mAChR/G-PROTEIN INTERACTIONS

Studies of chimeric receptors and deletion mutants of the mAChR subtypes have demonstrated that major sites for the interaction of mAChR subtypes with G proteins are contained in the cytoplasmic domains. In particular, the NH_2-terminal region of the third cytoplasmic loop of the mAChR subtypes (Fig. 1) appears to contain crucial determinants in allowing the receptors to recognize G proteins (33–37). The replacement of a 12-amino-acid segment in this region of the m1 mAChR with the corresponding peptide from the turkey β-adrenergic receptor resulted in muscarinic receptors that signaled like β-adrenergic receptors, i.e., they stimulated adenylyl cyclase via G_s (35). Studies with chimeras of the m2 and m3 mAChR showed that transfer of segments as small as 9–21 amino acids from this same region can specify an m2 mAChR to signal like an m3 mAChR and vice versa (37). However, additional portions of this loop as well as determinants in the second cytoplasmic loop also appear to play important roles (35, 36).

Other evidence that supports the concept that the third loop contains important structural information for signaling has been obtained from using antibodies and peptides (38). Antibodies prepared against synthetic peptides corresponding to various regions of the third cytoplasmic loops for the various mAChR subtypes are capable of selectively perturbing mAChR/G protein interactions. This was demonstrated using purified and reconstituted chick heart receptors and G proteins (38). When peptides corresponding to the NH_2-terminal region of the third loop of the m4 receptor were preincubated with purified G_o, they were found to block any subsequent interaction between the chick heart receptors and G_o (38).

REGULATION OF MUSCARINIC RECEPTOR SUBTYPES BY PHOSPHORYLATION-DEPENDENT PROCESSES

Like many other receptors, muscarinic receptors desensitize and down-regulate in response to prolonged agonist stimulation. It appears likely that there are diverse mechanisms involved in these processes. This is not surprising in view of the diversity of signaling events that are regulated by receptor activation. Direct and indirect evidence supports the concept that mAChR subtypes are regulated by multiple events involving protein phosphorylation, as suggested first by Burgoyne (39). Several groups have demonstrated that mAChRs in various cell types exhibit diminished activities in response to prolonged agonist activation, and in several cases this effect of agonist can be mimicked by activators of protein kinases (40–44). Notably, in several neuronal cell lines, activation of protein kinase C appears to have striking effects on mAChR function (reviewed in ref. 44).

Studies with avian and mammalian cardiac mAChRs have demonstrated that the mAChR are phosphorylated in an agonist-dependent manner in intact cells (45–47). In both cases, stimulation of ^{32}P-labeled tissue with a muscarinic agonist leads to increased phosphorylation of the mAChR in the intact cells (Fig. 3). The stoichiometry of phosphorylation is 3–5 mol phosphate/mol

Figure 3. Agonist-dependent phosphorylation of mAChR in intact chick heart cells. The figure shows an autoradiogram from an experiment where mAChR were purified by affinity chromatography from ^{32}P-labeled chick ventricular slices exposed to no stimulus (control, Con) or for 15 min to the agonists carbachol (Carb) or oxotremorine (Oxo). The band at 79 kDa corresponds to the purified chick heart mAChR. The extent of phosphorylation of 3–5 mol phosphate/mol protein; ~60% of the phosphate was incorporated into serine residues and ~40% into threonine residues (46).

receptor (after agonist treatment) and occurs on serine (60–70%) and threonine (30–40%) residues (46, 47). The chick heart preparation has been the most well studied because chick ventricle is a relatively rich and convenient source of mAChRs. In these cells, agonist-dependent phosphorylation of the receptors appears to correlate with a decreased affinity of the receptors for agonist and with a decreased ability of the receptors to produce a negative inotropic response (46). The time course and dose-response relationships for agonists to induce phosphorylation correlate with those that produce changes in receptor properties. Thus, at least in cardiac cells, mAChRs undergo phosphorylation in a manner that is consistent with the hypothesis that phosphorylation of these receptors may be an important reaction in the molecular events that result in desensitization. Similar studies of mAChR phosphorylation have not yet been carried out in other intact cell preparations. Studies of receptor phoshorylation with brain slices would be compromised by the known heterogeneity of mAChR in brain, whereas studies with neuronal cell lines have not been possible because most of these cells express an exceedingly small number of receptors (10–100 fmol/mg protein), making studies of protein phosphorylation extremely difficult. Studies of cells transfected with mAChR DNA and expressing high levels of mAChR should provide a fruitful approach.

PATHWAYS OF mAChR PHOSPHORYLATION: ROLE OF RECEPTOR-SPECIFIC KINASES

An obvious questions is: What are the pathway or pathways that lead to mAChR phosphorylation? Because the receptors couple to multiple effectors, many of which ultimately turn on or off phosphorylation-dependent events, one possibility is that the receptors are phos-

phorylated by protein kinases that are regulated by cascades triggered by mAChR activation. In chick cardiac cells, none of the common activators of "garden variety" protein kinases (in particular cyclic nucleotide analogs, activators of protein kinase C, a Ca^{2+} ionophore, or calmodulin inhibitors) cause phosphorylation of the receptors in the absence of a muscarinic agonist (46). Phosphorylation of mAChRs in cardiac cells is also not produced by activation of adenosine receptors, which in these cells couple to many of the same signal transduction pathways as the mAChRs. Taken together, these results suggest that either the mAChRs must be occupied by an agonist in order to serve as a substrate for phosphorylation (Fig. 4) and/or that the protein kinases commonly activated by signal transduction pathways do not participate in the agonist-dependent phosphorylation of the mAChRs. The latter possibility is difficult to directly test in intact cells, because if only the agonist-occupied receptors can act as substrates, then one would not observe phosphorylation of the receptors when activators of signaling pathways or of protein kinases are used in the absence of an mAChR agonist. If only agonist-occupied mAChR can serve as substrates for phosphorylation, the amount of phosphorylation observed will only be proportional to the amount of substrate (agonist-occupied receptor) present.

The agonist-dependent phosphorylation of mAChRs in intact cells is strikingly similar to that observed for several other members of the G protein-coupled superfamily of receptors. Most notably, the β-adrenergic receptors and rhodopsin undergo agonist-dependent phosphorylation in intact cells (for examples, see ref. 48). For these latter receptors, receptor-specific protein kinases that are referred to as the β-adrenergic receptor kinase (βARK) and rhodopsin kinase, respectively, have been identified and shown to phosphorylate their respective receptors in a manner that is highly agonist-dependent (see ref. 48 and references therein). No second messengers appear to be involved in these reactions. Rather, the phosphorylations appear to be highly dependent on conformational changes induced by agonist (norepinephrine or light). The agonist unoccupied, or antagonist-occupied, receptors are poor substrates for these enzymes. To determine whether similar reactions might explain the agonist-dependent phosphorylation of mAChRs, studies using purified mAChR reconstituted into detergent free liposomes have been useful. With such a strategy, mAChRs from chick heart act as excellent substrates in vitro for βARK (see Fig. 5 and ref. 49); in fact, the mAChR are phosphorylated as efficiently by βARK as are the adrenergic receptors. The phosphorylation of mAChRs by βARK occurs in a strictly agonist-dependent manner (Fig. 5) and with a stoichiometry and amino acid selectively that is strikingly similar to what is observed in agonist-stimulated intact cells. The mAChRs purified from porcine heart and brain have also been demonstrated to be substrates for what appears to be a βARK or βARK-like enzyme (the kinase was only partially pure and so its identity is not certain) (50).

Although it is tempting to speculate that βARK or a related kinase phosphorylates mAChRs in intact cells, further work is required to establish that this is so. In particular, recent studies have suggested that the ability of porcine brain and cardiac mAChRs to serve as substrates for the partially purified βARK-like enzyme could be modified by G proteins (51). If this is indeed due to

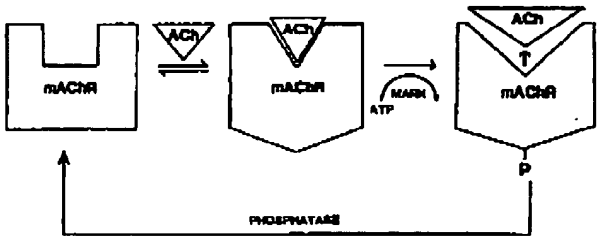

Figure 4. Model to describe the agonist-dependent phosphorylation of mAChR. In this model, based on results obtained with chick heart mAChR (46), agonist binding to the receptors induces a conformational change in the protein that allows them to become substrates for a muscarinic acetylcholine receptor kinase (MARK). Although no specific MARK has been identified, the β-adrenergic receptor kinase phosphorylates chick heart mAChR in an agonist-specific manner (49). The phosphatases that dephosphorylate the mAChR have not been identified.

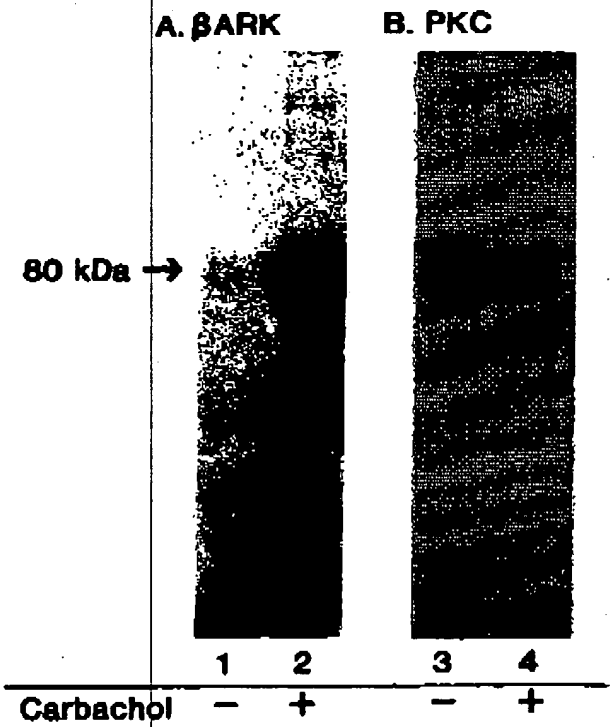

Figure 5. Agonist-dependent and -independent phosphorylation of chick heart mAChR in vitro by the β-adrenergic receptor kinase and protein kinase C. Autoradiogram depicting phosphorylated chick heart mAChR that were first purified and reconstituted into liposomes and then phosphorylated in vitro with purified β-adrenergic receptor kinase or protein kinase C in the presence or absence of the agonist carbachol. Note the marked agonist dependence of the βARK reaction, and in contrast, the agonist-independent phosphorylation by protein kinase C (replicated with permission from ref 52).

a reaction catalyzed by βARK, the situation would be different from the homologous system of the β-adrenergic receptors where no role of G proteins has been suggested in the modulation of phosphorylation. However, structural diversity may underlie interesting differences that may occur in the regulation of these related, yet distinct, G protein-coupled receptor systems. Of note in this regard is that for both the β-receptors and rhodopsin, the phosphorylations catalyzed by βARK and rhodopsin kinase, respectively, have been proposed to occur in the serine/threonine-rich COOH-terminal tails (see ref 48). Interestingly, none of the mAChR subtypes have serine/threonine-rich COOH-terminal tails (10–12). Instead, the only serine residues (which account for 60–70% of the phosphoamino acids in phosphorylated mAChR; refs 46, 47, 49, 52) available for phosphorylation in the various mAChR subtypes are found in the third cytoplasmic domains (Fig. 1). As these regions are involved in coupling the receptors to G proteins (33–38), it might not be surprising to find that sites for interactions with G proteins and sites for regulation by phophorylation might physically interact to modulate both the abilities of the mAChRs to activate G proteins and to undergo phosphorylation.

Another class of proteins plays a role in the regulation of the β-receptors and rhodopsin by phosphorylation. A protein known as arrestin for rhodopsin and β-arrestin for the β-adrenergic receptor appears to bind to the receptors only when they are phosphorylated and to modify their ability to interact with their respective G proteins (48). It is not yet known if similar proteins participate in the regulation of mAChR.

MUSCARINIC RECEPTORS ARE SUBSTRATES FOR MULTIPLE SECOND MESSENGER-ACTIVATED PROTEIN KINASES

Many cellular studies have suggested that mAChR subtypes might be directly regulated by protein kinase C(PKC) (44). In support of this concept, the purified chick heart mAChR (Fig. 5 and ref 52) and porcine cerebral mAChR (53) are excellent substrates for phosphorylation by purified PKC in vitro. The rate of phosphorylation of the avian heart mAChRs catalyzed by PKC is significantly more rapid than that catalyzed by βARK, whereas the stoichiometry and amino acid selectivity are similar (5 mol phosphate/mol receptor, predominately on serine residues) (52). Perhaps the most striking difference between the phosphorylation of mAChR catalyzed by βARK and by PKC is that, unlike the βARK mediated reactions, the PKC-mediated phosphorylation occurs in a totally agonist-independent manner (Fig. 5 and refs 52, 53). The lack of requirement for agonist occupancy for PKC-mediated phosphorylation suggests a potential role for PKC-mediated phosphorylation in the heterologous desensitization of mAChR (i.e., desensitization that is not initiated by the mAChR but by activation by another receptor of a common signaling pathway that may feed back on the mAChR).

The functional consequences of PKC-mediated phosphorylation have been studied initially in vitro and appear to involve modulation of mAChR/G protein interactions (52). However, an important caveat is that it is not yet known whether these reactions occur in intact cells, as other studies failed to reveal phosphorylation of mAChRs in intact chick heart cells after treatment with protein kinase C activators (46). Furthermore, it appears that mammalian cardiac receptors do not serve as substrates for PKC (43, 54). However, significant differences in avian and mammalian cardiac mAChRs have been noted (47). Further studies of phosphorylation of mAChR subtypes in intact cells are required to elucidate the role of protein kinase C in the regulation of these proteins.

Other studies have demonstrated that purified brain mAChR (likely a mixture of mAChR subtypes) (55) and porcine cardiac m2 mAChR (54) are substrates in vitro for cAMP-dependent protein kinase (PKA). Furthermore, another study suggested that PKA might modulate the ability of the m2 mAChR, but not the m1 mAChR, to serve as substrates for PKC (43). The physiological significance of these findings are not known because the activity of PKA should be attenuated by the activation of m2 and m4 receptors and remain unchanged after the activation of m1, m3, or m5 receptors. However, in some cells, vigorous activation of PI hydrolysis with concomitant increases in intracellular Ca^{2+} could lead to increases in cAMP as a result of activation of a Ca^{2+}/calmodulin-sensitive adenylyl cyclase.

WHICH MUSCARINIC RECEPTOR SUBTYPES ARE REGULATED BY PHOSPHORYLATION AND WHAT ARE THE FUNCTIONAL CONSEQUENCES?

An important reminder in considering the regulation of mAChR by phosphorylation is that, so far, almost all studies demonstrating that receptor phosphorylation occurs in intact cells have used the chick heart as a model system. However, it is still unclear as to which mAChR subtypes are expressed and regulated by phosphorylation in chick heart. It is possible that more than one mAChR subtype may be expressed in avian cardiac cells, in contrast to mammalian cardiac cells that appear to express only the m2 type. An avian m2 mAChR likely exists in chick heart as an m2 cDNA has recently been isolated and sequenced from a chick heart cDNA library (56, and K. Wortley and M. M. Hosey, unpublished results); however, chick heart also may express an avian m4 mAChR, as suggested by results of Northern analyses of chick heart mRNA with an avian m4 probe (56). Thus, despite the utility of the chick heart model, it has not yet provided unequivocal answers to the question of which mAChR subtypes are regulated by phosphorylation.

The obvious approach to understanding subtype-specific regulation of mAChR is to use cells transfected with the cDNAs for the mAChR subtypes. This approach can provide cells uniquely expressing a single mAChR subtype, and through the use of different expression systems, the level of expression can be enhanced to facilitate studies of the phosphorylation of the proteins. A caveat to such studies is that not all the receptors in cell lines over-expressing the receptors may be appropriately coupled to G proteins and/or signal transduction pathways. This could complicate studies that seek to correlate receptor phosphorylation with desensitization and down-regulation. The future promises to be an exciting time for unraveling the molecular basis of desensitization of mAChR subtypes and for understanding the roles that phosphorylation by receptor-specific and ge-

neric protein kinases have in regulating the activities of the diverse and highly interesting mAChR subtypes and related G protein-coupled receptors. FJ

The work cited from the author's laboratory was supported by National Institutes of Health grant HL 31601 and by fellowships from the American Heart Association of Chicago to M. Kwatra, R. Richardson, and K. Wortley. I thank my colleagues Debbie Birn, Dick Green, Madan Kwatra, Eddie Leung, Shekhar Mayanil, Judy Prasienski, Richardo Richardson, and Kathy Wortley for their efforts in attempting to unravel the mysteries relating to regulation of mAChR subtypes by phosphorylation. I thank a wintry day in Chicago and a bout with the common cold for the uninterrupted time to write this review.

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